Molecular Phylogenetics and Evolution
Vol. 22, No. 3, March, pp. 364–374, 2002
doi:10.1006/mpev.2001.1069, available online at http://www.idealibrary.com on IDEAL®

Molecular Phylogeny of the Snubnose Darters, Subgenus *Ulocentra* (Genus *Etheostoma,* Family Percidae)

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Received September 26, 2000; revised September 14, 2001

Snubnose darters comprise one of the largest subgenera of the percid genus *Etheostoma*. Many species are described based on differences in male breeding coloration. Few morphological synapomorphies have been proposed for the subgenus and their relatives, making it difficult to delineate monophyletic clades. The phylogenetic relationships of the 20 snubnose darter species of the subgenus *Ulocentra* and 11 members of its proposed sister subgenus *Etheostoma* were investigated with partial mitochondrial DNA sequences including 1033 bp encompassing the entire mitochondrial control region, the tRNA-Phe gene, and part of the 12S rRNA gene. Two hypotheses on the relationship and monophyly of the two subgenera were evaluated. Both maximum-parsimony and neighbor-joining analyses supported monophyly of the subgenus *Ulocentra* and resolved some species-level relationships. The banded darter, *E. zonale,* and its sister taxon, *E. lynceum,* were not closely related to the snubnose darters and appear to be diverged from the other members of the subgenus *Etheostoma,* fitting their former distinction as the recognized subgenus *Nanostoma*. The sister group to *Ulocentra* appears to be a restricted species assemblage within the subgenus *Etheostoma* containing *E. blennioides, E. rupestre, E. blennius,* and the *E. thalassinum* species group. The placement of the harlequin darter, *E. histrio,* is problematic, and it may represent a basal member of *Ulocentra* or of the restricted subgenus *Etheostoma*. Despite recent estimates of divergence times between nominal *Ulocentra* taxa, each species exhibits its own unique set of mtDNA haplotypes, providing no direct evidence for current genetic exchange between species. The nominal taxa of snubnose darters thus appear to be evolving independently from each other and therefore constitute valid species under the Phylogenetic Species Concept.

Key Words: **mtDNA; control region; tRNA-Phe; 12S rRNA; species concepts; molecular systematics; Perciformes; Teleostei; *Nanostoma*.**

INTRODUCTION

During the "Golden Era" of darter taxonomy (1841–1897), nearly 100 new species of North American darters were described and a large number of darter genera were designated without careful consideration of the underlying relationships of these fishes (Collette, 1967). R. M. Bailey reorganized darter nomenclature by reducing the number of genera to three (*Percina, Ammocrypta,* and *Etheostoma*) and subsuming many previous genera to subgeneric status (Bailey *et al.*, 1954; Bailey and Gosline, 1955). The genus *Etheostoma* is currently subdivided into 17 or 18 subgenera, with approximately 120 taxa, making it the largest genus of North American freshwater fishes. Members of the subgenus *Ulocentra,* termed snubnose darters, have attracted recent interest, numbering 20 described species and several undescribed forms. However, species distinction within *Ulocentra* is questionable due to morphological overlap in meristic morphological characters (Page and Burr, 1982; Suttkus and Etnier, 1991; Suttkus and Bailey, 1993) and inability to resolve them in principal component analyses (Boschung *et al.*, 1992; Suttkus *et al.*, 1994). Species descriptions have relied on variation in male color patterns which develop during a 3-month breeding season and are obscurred by cryptic coloration during the remainder of the year.

Despite apparent morphological similarities among *Ulocentra* species, few synapomorphies unite the subgenus, raising questions about its monophyly. Although recent diagnoses and redescriptions of the subgenus *Ulocentra* have been performed on a limited number of snubnose species (Bouchard, 1977; Bailey and Etnier, 1988), the consideration of all 20 species reveals no morphological synapomorphies for the subgenus. The behavioral character proposed by Bailey and Etnier (1988) of females laying eggs individually on vertical rock faces is a possible synapomorphy, but the reproduction of only 9 species has been studied to date (Winn 1958a,b; Stiles, 1974; O'Neil, 1981; Page and Mayden, 1981; Page *et al.*, 1982; Page, 1985; Carney and Burr, 1989; Keevin *et al.*, 1989; Weddle, 1990;

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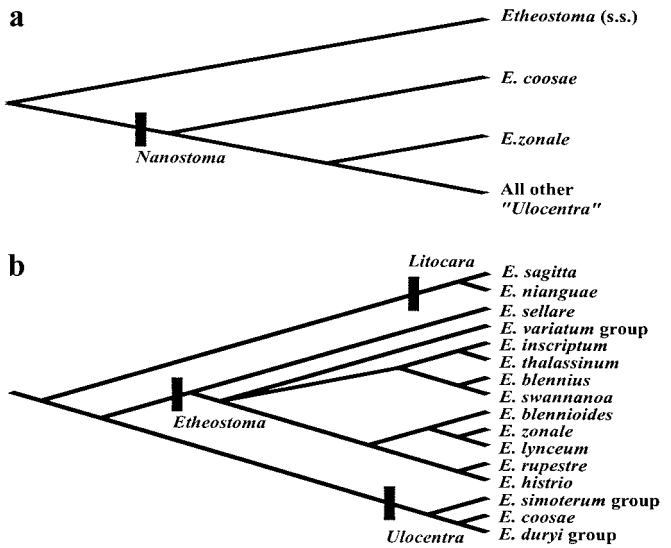

FIG. 1. Two proposed hypotheses on the phylogenetic associations between species in the subgenera *Etheostoma* and *Ulocentra*; (a) from Page (1981); (b) from Bailey and Etnier (1988).

Bauer *et al.*, 1995; Johnston and Haag, 1996; Porterfield, 1997). The subgenus *Etheostoma*, with 15 nominal species, has been proposed as the sister subgenus to *Ulocentra* (Collette, 1962; Richards, 1966; Bouchard, 1977; Bailey and Etnier, 1988) but the lack of synapomorphies is problematic.

Two competing hyptheses have been proposed with regard to the phylogenetic relationships of species in the subgenera *Etheostoma* and *Ulocentra*: (1) several systematists (Page, 1981; Page and Burr, 1982; Burr and Warren, 1986) regard the banded darter, *E. zonale*, as more closely related to the snubnose darters than to members of the subgenus *Etheostoma* and group the snubnose darters under the subgeneric name *Nanostoma* (Fig. 1a) and (2) Bailey and Etnier (1988) relegate the morphological resemblance between *E. zonale* and *Ulocentra* to evolutionary convergence and place *E. zonale* within the subgenus *Etheostoma* as the sister subgenus to *Ulocentra* (Fig. 1b).

In this study, competing hypotheses about the phylogenetic relationships of the snubnose darters are evaluated using DNA sequences from the alignable portion of the mtDNA control region (830–836 bp), the tRNA Phenylalanine (Phe) gene (68 bp), and part of the 12S rRNA gene (120 bp). Species-level relationships and population structure of taxa of *Ulocentra* are also investigated and interpreted under the Biological, Monophyletic, Genealogical, and Phylogenetic Species Concepts.

MATERIALS AND METHODS

Samples of the 20 species of *Ulocentra* and 11 species of possibly related darters were collected by seining from 62 localities, as indicated in Table 1 and Fig. 2. Samples are vouchered in The Ohio State University Museum of Zoology (Table 1). Genomic DNA was extracted from muscle and fin tissue following standard protocols (Maniatis *et al.*, 1982; Zhang and Tiersch, 1994). Mitochondrial DNA from the tRNA phenylalanine to the 12S RNA gene (about 1600 bp) was amplified using the polymerase chain reaction (PCR) from 100–1000 ng of the genomic template in 50-μl reactions using 2.5 mM $MgCl_2$, 2 mM each dNTP, 0.5 units *Taq* DNA polymerase, 5 pM each of a pair of oligonucleotide primers LPro and 12Sa-rev (Table 2), and reaction buffer (Promega or Gibco BRL) to a final 1X concentration. PCR amplifications were conducted in a Perkin Elmer Cetus DNA Thermal Cycler under the following profile: 2 min hot start at 93°C, followed by 32 cycles of 45 s at 93°C, 1 min at 58°C, and 2 min at 72°C.

PCR products from several species were cloned (TA Cloning Kit; Invitrogen Corp., LaJolla, CA) and sequenced (Sequenase; USB, Cleveland, OH) using the universal primers for the vector. From these initial sequence data, nine additional internal primers were designed specifically for darters in conserved regions of the sequences (Table 2). Internal primers were end-labeled with [^{32}P]ATP to facilitate sequencing with the dideoxy chain-termination procedure (dsDNA Cycle Sequencing System; Gibco BRL). Sequence reactions were run for 3000 to 12,000 V-h through 6–8% polyacrylamide gels and visualized by autoradiography.

Intraspecific variation was investigated in 16 select darter species using a heteroduplex visualization method (Porter, 1999) to screen darter populations for sequence variation. A 355-bp section of the left domain of the mtDNA control region (excluding the hypervariable 10-mer repeated portion) was amplified by PCR with primers DHET.1 and TDKD (Table 2), with an annealing temperature of 60°C and a final extension step of 10 min at 72°C. A mixture of the amplification product with a known sequenced product (referred to as the driver) was then created. The concentration of DNA in the PCR amplifications was measured by spectroscopy to assure the equal mixing of 2.5 μg of driver with 2.5 μg of test sample DNA in a final volume of 15 μl of 5 mM EDTA. The DNA mixtures were heated to 95°C, cooled, and electrophoresed through a vertical SequaGel MD following the manufacturer's directions (National Diagnostics Inc., Atlanta, GA). All samples producing heteroduplexes were tested in subsequent runs against each other, until the number and frequency of each haplotype was determined for the population. A representative of each haplotype was then amplified with the LPRO and 12Sa-rev primers and sequenced in its entirety following the procedure outlined above.

DNA sequences were aligned to previously determined percid (GenBank sequences U90617–U90624;

TABLE 1

Taxa, Site Numbers (in Reference to Fig. 2), Locality, OSUM Catalogue Numbers, and GenBank Accession Numbers

Species	Site	Collection locality	OSUM	GenBank
E. atripinne	12	Duck River, Marshall Co., TN	76967	AF404582
	34	Bledsoe Creek, Sumner Co., TN	85566	AF404581
	90	Marrowbone Creek, Cheatham Co, TN	95152	AF404583
E. baileyi	52	Little Sexton Creek, Clay Co., KY	95138	AF404588
	70	Clear Creek, Rockcastle Co., KY	85580	AF404589
E. barrenense	2	Little Trammel Creek, Allen Co., KY	95115	AF404584
E. bellator	33	Gurley Creek, Blout Co., AL	85432	AF404557
	50	Murphy Creek, Blount Co, AL	95139	AF404556
E. blennioides	11	Mill Creek, Putnam Co., TN	95136	AF404525
	63	Pomme de Terre River, Polk Co., MO	85512	AF404524
	86	Big Darby Creek, Franklin Co., OH	95146	AF404523
	88	Rocky River, Cuyahoga Co., OH	95109	AF404526
E. blennius	37	McWilliams Creek, Sequatchie Co., TN	95143	AF404528
	84	Indian Creek, Hardin Co., TN	95155	AF404529
E. brevirostrum	30	Shoal Creek, Cleburne Co., AL	95069, 95204	AF404566, 70
	75	Mountaintown Creek, Gilmer Co., GA	95205	AF404568
	76	Conasauga River, Polk Co., TN	95209	AF404571
	77	Etowah River, Dawson Co., GA	95206	AF404569
	78	Cochrans Creek, Dawson Co., GA	95110	AF404567
E. chermocki	31	Turkey Creek, Jefferson Co., AL	85462	AF404558
	32	Turkey Creek, Jefferson Co., AL	85401	AF404559
E. colorosum	46	Jordan Creek, Conecuh Co., AL	85377	AF404542
	51	Pine Barren Creek, Escambia Co., FL	85559	AF404541
E. coosae	27	Lake Creek, Floyd Co., GA	85143	AF404535
	30	Shoal Creek, Cleburne Co., AL	95203, 95202	AF404531, 32,
			95202, 95202	AF404533, 34
	76	Conasauga River, Polk Co., TN	NA	AF404538
	81	Mosley Springs, Chatooga Co., GA	95200	AF404536
	82	Minnewauga Creek, Polk Co., TN	95201	AF404537
E. duryi	37	McWilliams Creek, Sequatchie Co., TN	85726	AF404560
	38	Running Water Creek, Marion Co., TN	85286	AF404561
	58	Cane Creek, Lincoln Co., TN	95196	AF404562
E. etnieri	11	Mill Creek, Putman Co., TN	85239	AF404573
	61	Cherry Creek, White Co., TN	95111	AF404572
E. flavum	5	Pleasant Run, Logan Co., KY	95128	AF404565
	8	Pleasant Run, Logan Co., KY	95124	AF404563
	13	Defeated Camp Creek, Hickman Co., TN	77772	AF404564
E. histrio	79	Trout Creek, LaSalle Co., LA	95149	AF404530
E. inscriptum	68	Little Eastatoe Creek, Pickens Co., SC	85595	AF404522
E. lachneri	44	Wolf Creek, Choctaw Co., AL	77735	AF404550
	71	Elliotts Creek, Hale Co., AL	85527	AF404551
E. lynceum	16	Clear Creek, Henry Co., TN	95108	AF404519
	43	Pumpkin Creek, Lafayette Co., MS	85382	AF404518
E. pyrrhogaster	16	Clear Creek, Henry Co., TN	76981	AF404547
	17	Terrapin Creek, Henry Co., TN	76946	AF404546
E. rafinesquei	4	Wiggington Creek, Logan Co., KY	95725	AF404585
	69	Barren Run, LaRue Co., KY	85609, 85609	AF404586, 87
E. ramseyi	45	Little Creek, Merengo Co., AL	85362	AF404554
	56	Cahaba River, Jefferson Co., AL	95137	AF404555
E. raneyi	41	Hurricane Creek, Lafayette Co., MS	95197	AF404552
	42	Graham Mill Creek, Lafayette Co., MS	95198	AF404553
E. rupestre	44	Wolf Creek, Choctaw Co., AL	95140	AF404527
E. scotti	24	Butler Creek, Cobb Co., GA	85164, 85299	AF404539, 40
E. simoterum	9	Caney Valley Creek, Claiborne Co., TN	95122	AF404578
	10	Little Sycamore Creek, Claiborne Co., TN	76862	AF404574
	12	Duck River, Marshall Co., TN	76967	AF404575
	53	Little River, Blount Co., TN	85230	AF404577
	57	North Fork Blue Creek, Giles Co., TN	95133	AF404576
	65	W. Prong Little Pigeon River, Sevier Co., TN	84456	AF404580
	74	Clifty Creek, Morgan/Roane Co., TN	95151	AF404579

TABLE 1—*Continued*

Species	Site	Collection locality	OSUM	GenBank
E. swannanoa	65	W. Prong Little Pigeon River, Sevier Co., TN	84457	AF404520
E. tallapoosae	40	Jumpin In Creek, Carroll Co., GA	77719	AF404545
	48	Enitachopco Creek, Clay Co., AL	85254	AF404544
	49	Verdin Creek, Cleburne Co., AL	95199	AF404543
E. tetrazonum	63	Pomme de Terre River, Polk Co., MO	85516	AF404512
E. thalassinum	66	S. Saluda River, Pickens/Greenville Co., SC	85591	AF404521
E. variatum	7	Gladie Creek, Menifee Co., KY	95105	AF404514
	87	Big Darby Creek, Pickaway Co., OH	95147	AF404513
E. zonale	2	Little Trammel Creek, Allen Co., KY	95120	AF404516
	6	Red River, Powell/Menifee Co., KY	95130	AF404517
	85	Big Walnut Creek, Franklin Co., OH	95210	AF404515
E. zonistium	14	Sycamore Creek, Benton Co., TN	77756	AF404548
	15	West Sandy Creek, Henry Co., TN	76871	AF404549

Faber and Stepien, 1997) sequences using the Eyeball Sequence Editor, ESEE3S ver.3.0s (Cabot and Beckenback, 1989). The aligned sequence data from the control region, tRNA-Phe gene, and partial 12S rRNA gene (aligned GenBank entries AF404512–AF404589) were combined and analyzed using two methods of phylogenetic reconstruction. Maximum-parsimony (MP) analysis was performed using HENNIG86 version 1.5 (Farris, 1988) with a branch and bound search. Relative support of the data set for the nodes was evaluated using NONA version 1.16 (Goloboff, 1993) and 1000 randomly seeded replicates. Bremer support for the nodes were calculated in NONA based on 10,510 trees up to 5 steps away from the most parsimonious trees. Additional support for the MP nodes was investigated with 10,000 jackknife replicates in Random Cladistics version 2.1.1 (Siddall, 1996) using the program JACK. Alternative topologies were analyzed in CLADOS version 1.4.95 (Nixon, 1993) to calculate the number of additional evolutionary steps required for alternative hypotheses. A pairwise genetic distance matrix was generated under the Kimura two-parameter models using MEGA version 1.01 (Kumar *et al.*, 1993) and a neighbor-joining (NJ) tree was constructed with 1000 bootstrap replicates. Examinations of substitution patterns were conducted by plotting pairwise frequencies of transitions (TS) and transversions (TV) against pairwise nucleotide sequence divergence to evaluate the potential for saturation.

RESULTS

Sequencing supplemented by heteroduplex analysis revealed 78 distinct mtDNA haplotypes within the 225 assayed specimens representing 20 species of *Ulocentra* and 11 species of *Etheostoma*. Aligned sequence data from the mtDNA control region, tRNA-Phe gene, and partial 12S rRNA gene were combined into a single data set comprising 1033 bp for phylogenetic analysis since mtDNA is a single locus. *Etheostoma tetrazonum* was used as the outgroup for the 78 taxa following its presumed ancestral position (McKeown *et al.*, 1984). The data set contained 284 variable sites, 224 of which were informative for MP. The hypervariable portion of the control region, consisting of a 10-mer tandem repeat element and an imperfect degraded repeat section (see Faber and Stepien, 1997), was excluded from phylogenetic analysis due to potential problems in identifying homology. This region may be of interest in future studies on intraspecific variation in darters.

An analysis of the distribution of variable sites across the sequence revealed that the control region is evolving 1.5 to 1.75 times faster than the two coding genes as reported by Avise *et al.* (1987) for other species. The elevated rate of molecular evolution in the control region is expected, given the importance of secondary structure to the two coding RNA genes versus the noncoding control region.

A saturation test on the combined data indicated that transversions slightly outnumbered transitions with an overall ratio of 1.25:1 (Fig. 3), similar to the results found by Turner (1997) in an examination of 33 darter species with a partial (366-bp region of the left domain) control region data set. The pairwise plot of observed TV versus sequence divergence did not deviate substantially from a linear fit, but observed TS decreased above the 4.5% nucleotide divergence level, indicating some saturation effect for distantly related taxa.

Ten equally parsimonious trees with 808 steps were obtained from the branch and bound MP search and their strict consensus tree is shown in Fig. 4, with Bremer support and jackknife values above 50% indicated. Taxa comprising the subgenus *Ulocentra* form a weakly supported monophyletic group containing two major subgroups (also weakly supported). A larger clade, containing *E. histrio,* the *E. thalassinum* species group sensu Richards (1966), and the three species *E. rupestre, E. blennius,* and *E. blennioides,* forms the sister clade to the subgenus *Ulocentra*. The weak support for the *Ulocentra* clade and the restricted subgenus *Etheostoma* clade results from the problematic

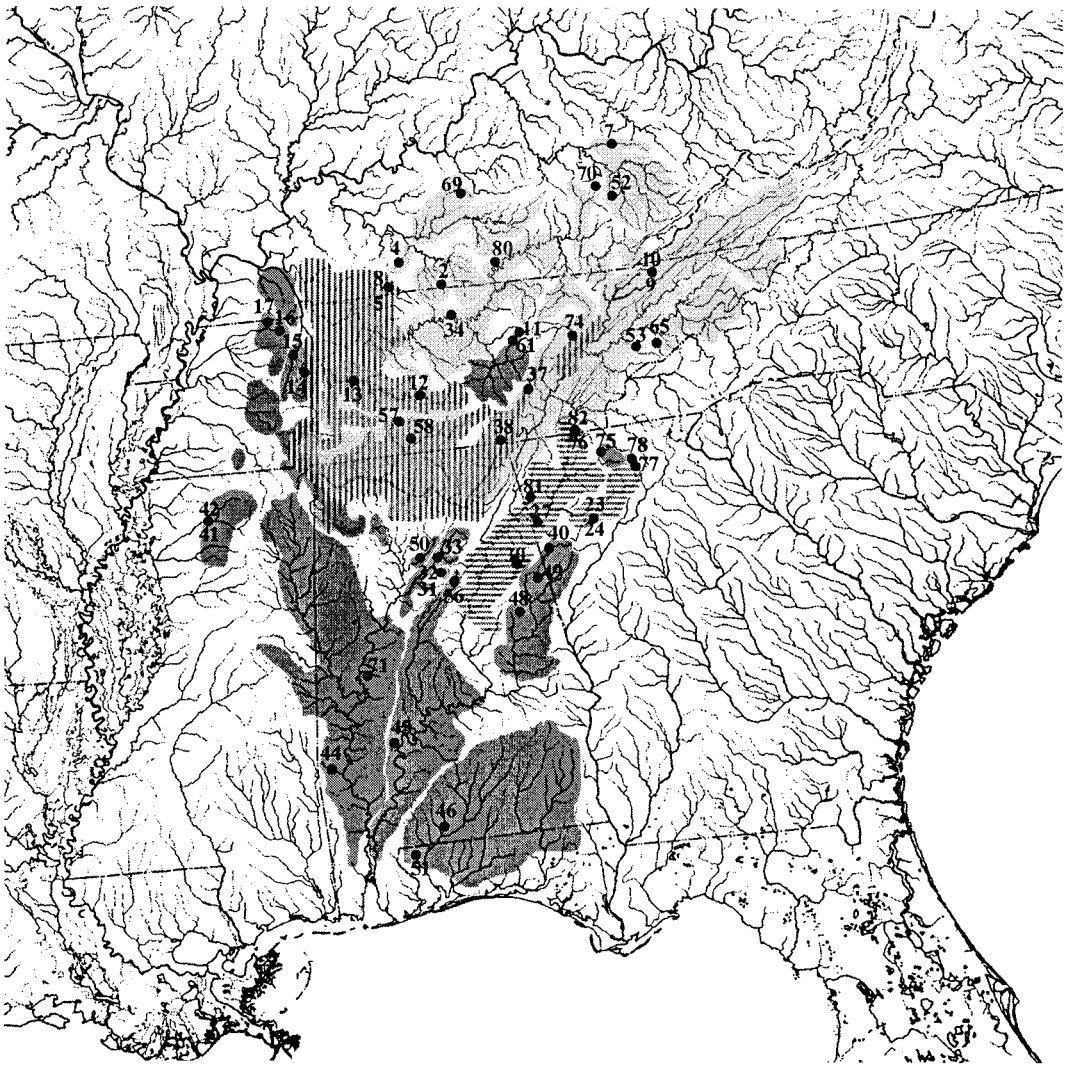

FIG. 2. Distribution of the 20 described species of the subgenus *Ulocentra*. The four major clades of *Ulocentra* are composed of the *E. simoterum* group (light gray), the *E. duryi–E. flavum* clade (vertical bars), the *E. coosae–E. scotti* clade (horizontal bars), and the remaining members of the *E. duryi* species group (dark gray). Dots indicate sample localities with collection site numbers (see Table 1 for collection localities).

placement of *E. histrio,* which appears basal to the other member. Constraining the tree to include *E. histrio* as the basal member of *Ulocentra* requires 2 additional evolutionary steps. Two basally located outgroups, one containing *E. zonale* and *E. lynceum* and the other containing *E. variatum,* robustly cluster outside the other members of the subgenus *Etheostoma*. Constraining the tree to the topology of Page (1981) or any other inclusion of the *E. zonale–E. lynceum* clade as outgroups to, or as a clade within, *Ulocentra* requires 9 to 24 additional evolutionary parsimony steps.

The NJ phenogram (Fig. 5) is largely consistent in topology with the MP analysis. Taxa comprising the subgenus *Ulocentra* form a unified assemblage with congruent substructure as seen with MP. However, the NJ tree depicts *E. histrio* as the basal member of the *E. simoterum* group with low bootstrap support. A second assemblage containing members of the *E. thalassinum* species group plus *E. rupestre, E. blennius,* and *E. blennioides* forms a sister assemblage to the *Ulocentra* + *E. histrio* group, as in MP analysis. Two progressive outgroups, one formed by *E. zonale–E. lynceum* samples and the other by the two *E. variatum* samples, cluster away from the other members of the subgenus *Etheostoma,* resulting in the largest branch lengths on the phenogram.

DISCUSSION

Systematics and Classification

Both methods of phylogenetic reconstruction revealed a unified *Ulocentra* assemblage sensu Bailey and Etnier (1988), as the sister taxon to a restricted

TABLE 2

Primers Used in PCR, Heteroduplex, and Sequencing of the mtDNA CR, tRNA Phe Gene, and Partial 12S rRNA Gene in Darters

Primer	Sequence	Direction and use
LPro	5′AACTCTCACCCCTAGCTCCCAAAG3′	Light PCR primer
DHET.1	5′ACACCATACATTTATATTAACCAT3′	Light heteroduplex primer
BAPD.1	5′ATCTCGTCATACCTCAAAATCTT3′	Light sequencing primer
BAPD.2	5′ACGGTTATTGAAGGTGAGGGAC3′	Light sequencing primer
TDKD	5′CCTGAAGTAGGAACCAGATG3′	Heavy sequencing primer/heteroduplex primer
BAPD.3	5′GAACCACATATTAGGATATCATG3′	Light sequencing primer
BAPD.4F	5′TGAAAACCCCCCGGAAACAGG3′	Light sequencing primer
TPhen R	5′CTAGGGCCCATCTTAACATCTTCAG3′	Heavy sequencing primer
12S.1-rev	5′GGGTGTGGCTTAGCAAGGCGT3′	Heavy sequencing primer
12S.1	5′GCCTAGCCACACCCCCACGG3′	Light sequencing primer
12S.2	5′GGTCAATTTCGTGCCAGCCA3′	Light sequencing primer
12Sa-rev	5′TAGTGGGGTATCTAATCCCAG3′	Heavy PCR primer
H12S-rev	5′GACATCCCGTAAGAGTGCCCC3′	Heavy PCR primer

subgenus *Etheostoma* containing *E. blennioides–E. blennius–E. rupestre* and the *E. thalassinum* species group. The hypothesis that *E. zonale–E. lynceum* are the closest relatives to snubnose darters (Page, 1981) is refuted, as neither species forms a monophyletic clade with *Ulocentra* without including the more immediate relatives of the restricted subgenus *Etheostoma*. Furthermore, a tree constraining the *E. zonale–E. lynceum* clade as a direct outgroup of (or as a clade within) *Ulocentra* requires a minimum of nine extra evolutionary steps. The distant relationships of the *E. variatum* species group and *E. zonale–E. lynceum* to the members of the restricted subgenus *Etheostoma* is shown by the long branches on the phenogram (Fig. 5). Monophyletic subgenera would be obtained by restricting the subgenus *Nanostoma* (Putnam *in* Jordan 1877) to include only *E. zonale* and *E. lynceum*, as suggested by Clayton (1984), and resurrecting the subgenus *Poecilichthys* Agassiz (1854) to include the members of the *E. variatum* species group.

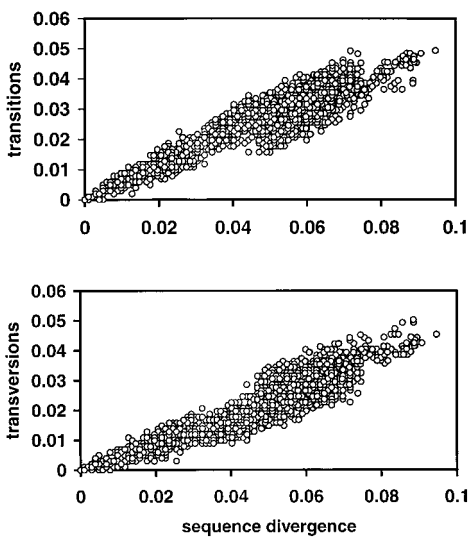

FIG. 3. Frequencies of pairwise observed transitions (top) and transversions (bottom) plotted against nucleotide sequence divergence.

The subgenus *Ulocentra* contains two species groups consistent with the arrangement of Bailey and Etnier (1988). The MP cladogram places *E. baileyi* as the basal member of the *E. simoterum* group. There are two basal subgroups within the *E. duryi* species group, one consisting of *E. coosae–E. scotti* and the other of *E. duryi–E. flavum*. The molecular analyses do not definitively resolve which of these two subgroups forms the base of the *E. duryi* species group (Fig. 4). The remaining members of the *E. duryi* species group form a well-supported clade. The "*E. tallapoosae*" subgroup *sensu* Suttkus *et al.* (1994) is paraphyletic without the inclusion of the other coastal plains species *E. bellator*, *E. chermocki*, and *E. brevirostrum*.

Legitimacy of the Described Ulocentra Species

Most species of *Ulocentra* are allopatric, and the few species with sympatric distributions belong to separate clades (Fig. 2). However, the sister taxa *E. simoterum–E. atripinne* are both present in the Duck and Cumberland drainages and exhibit clinal gradients in morphological characters (Bouchard, 1977; Etnier and Starnes, 1993). We analyzed both types in sympatry from the Duck River, which were distinguishable by dorsal saddle pattern and mtDNA haplotypes. Additional sample sites and sequences are desirable to test their possible species-level seperation.

Hybridization between the sister species *E. duryi–E. flavum* has been documented in a small region of sympatry within the headwaters of the Duck River system (Etnier and Bailey, 1989). Hybrids between the more distantly related species *E. brevirostrum–E. coosae*

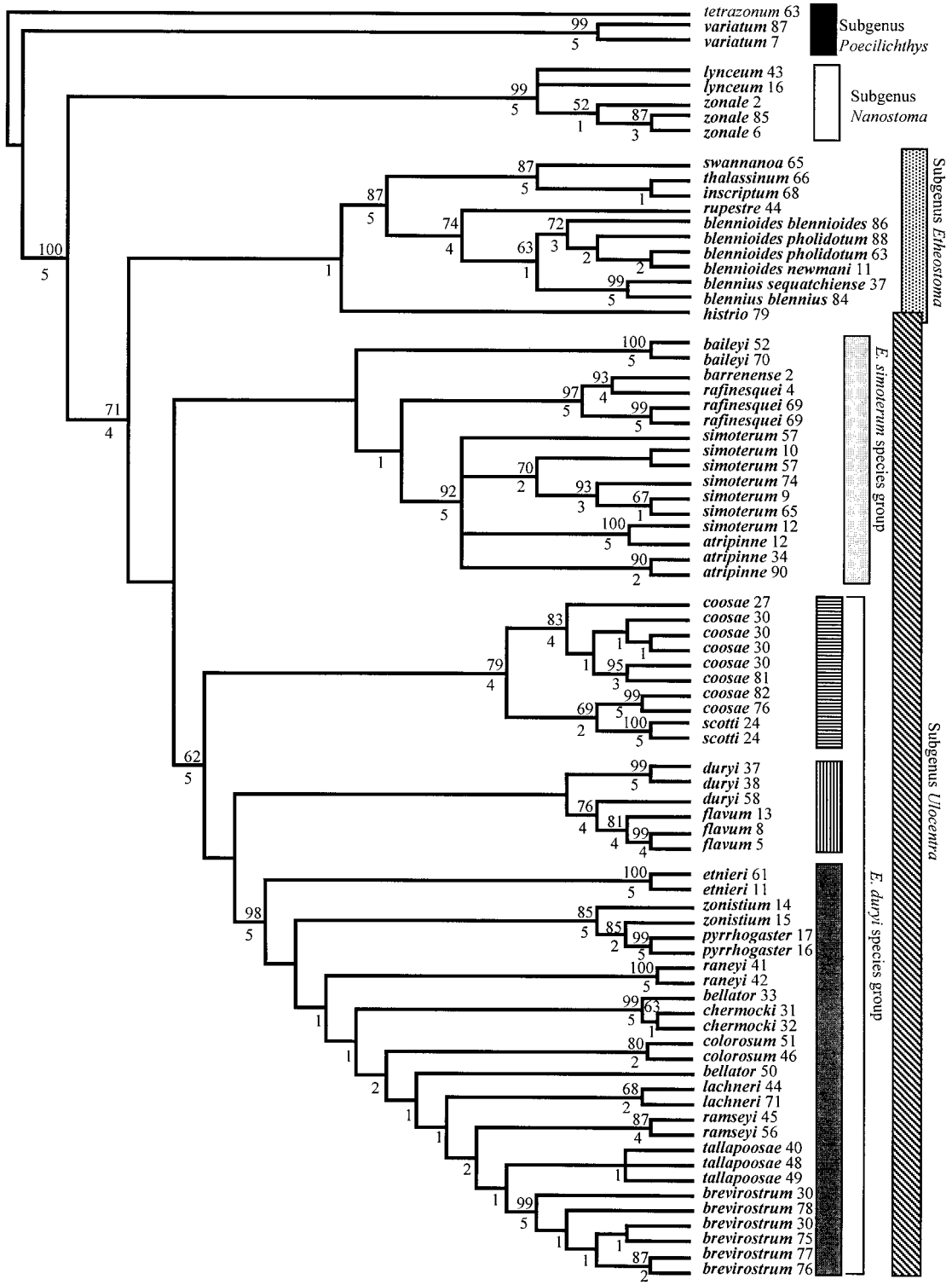

FIG. 4. Maximum-parsimony strict consensus cladogram of 78 darter taxa representing the subgenera *Etheostoma* and *Ulocentra* as inferred from mtDNA sequence data. Support for clades are shown with jackknife values (above the internodes) and Bremmer support values (below the internodes). Taxon names are followed by the collection site number.

(N. M. Burkhead, personal communication), which are members of two major separate clades, have been observed in the Upper Coosa System. Aquarium trials by Winn (1958a) placed reproductively active individuals of *E. rafinesquei* and *E. barrenense* together and noted hybrid spawning resulting in fertilized eggs in these

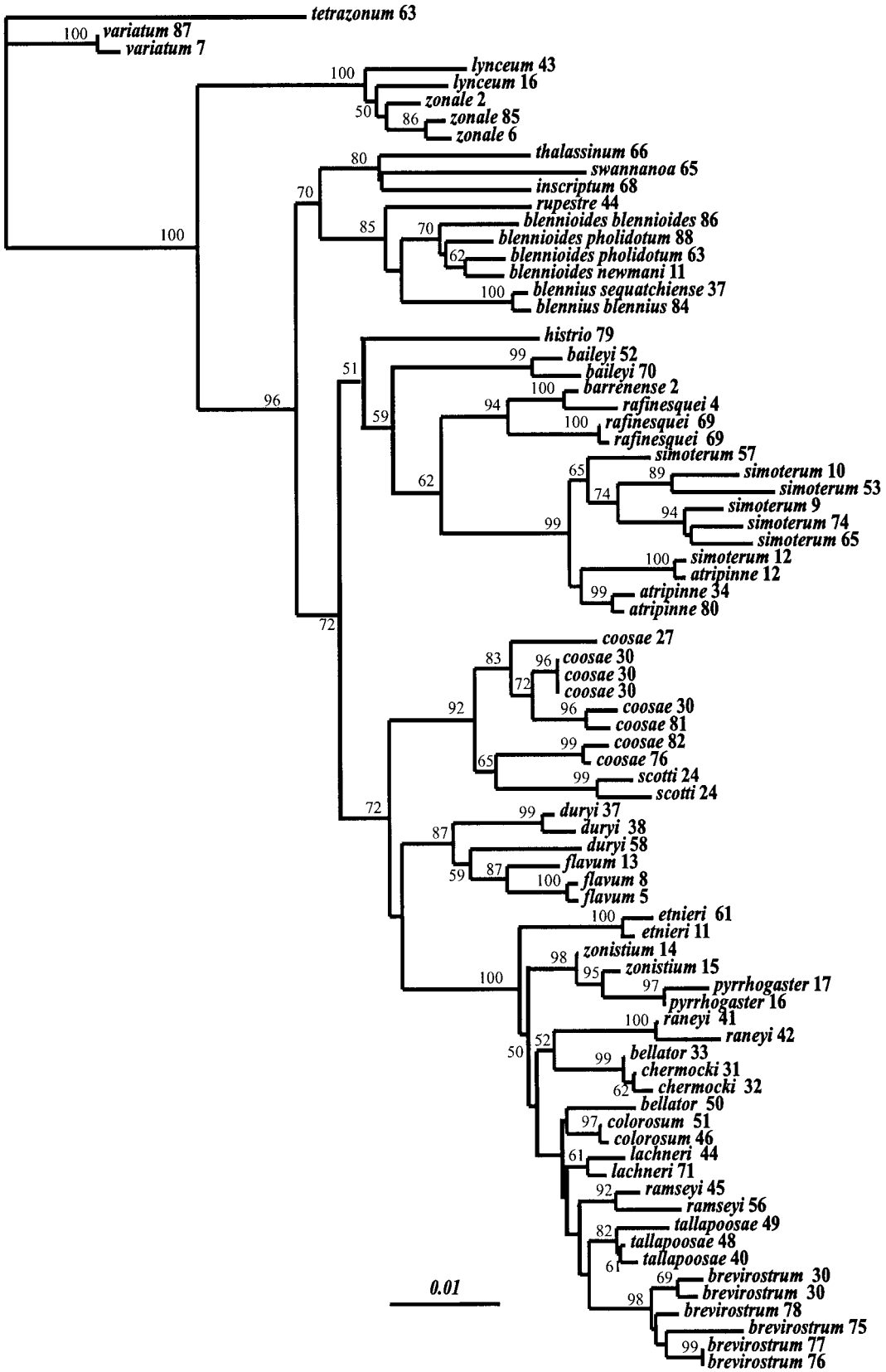

FIG. 5. Neighbor-joining phenogram of 78 darter taxa representing the subgenera *Etheostoma* and *Ulocentra* as inferred from mtDNA sequence data. Support for groups is shown with bootstrap values from 1000 replicates. Taxon names are followed by collection site numbers.

normally allopatric sister species. These observations collectively indicate that species isolating mechanisms within the snubnose darters are weak and that the species diversity is maintained primarily through allopatry. Given these observations and results, a strict interpretation of the Biological Species Concept (Dobzhansky, 1937) would results in the recognition of only four "legitimate" polychromatic species, represented by the four major clades delineated in Fig. 2.

The mtDNA sequence data suggest that the warrior darters of the *E. chermocki* species group (*E. chermocki* and *E. bellator*) are a polyphyletic assemblage. An allozyme study on the *E. chermocki* group (Clabaugh *et al.*, 1996) also found *E. bellator* to be polyphyletic and did not detect significant allelic differences between samples from Gurley Creek (middle Locust Fork) and the type locality (Murphy Creek–Mulberry Fork). The finer resolution of mtDNA sequence data and the additional outgroups used in this study reveal that samples of *E. bellator* from Gurley Creek are a sister taxon to *E. chermocki,* while those from the type locality for *E. bellator* are more closely related to other snubnose taxa. Additional molecular and morphological studies are needed to resolve these problems.

The MP analysis (Fig. 4) reveals that most sister species groups (*E. coosae–E. scotti, E. rafinesquei–E. barrenense, E. pyrrhogaster–E. zonistium, E. dury–E. flavum,* and *E. simoterum–E. atripinne*) are not reciprocally monophyletic. If the Monophyletic Species Concept (Donoghue, 1985; Mishler 1985) or the Genealogical Species Concept (Baum, 1992; Davis, 1997) is strictly applied to the subgenus *Ulocentra,* only 10 of the 20 described species would be valid. Given the recent radiation of the group from four independently evolving clades, it is likely that many of the sister species pairs have not yet undergone the process of complete stochastic lineage sorting as described in Avise (1994, pp. 126–133; 2000) to render them monophyletic, even though heteroduplex and sequence analyses indicate that these sister pairs no longer exchange genes. Nuclear DNA sequence data are needed to more fully address this problem.

The process of mtDNA lineage sorting has been modeled with respect to the size of the founder population (Avise *et al.*, 1984). While a small founding population of around 10 individuals may only take 10^2 generations to purge itself of two or more haplotypes, a population of 10,000 individuals may take up to 10^5 generations to do so (Avise *et al.*, 1984). Snubnose darters are usually among the most abundant species at a stream site and have a generation time of 1 to 2 years (Page and Mayden, 1981; O'Neil, 1981; Clayton, 1984; Carney and Burr, 1989). Even if the population sizes of ancestral stocks for snubnose sister species were small, around 50 individuals, it would take an estimated 5000–10,000 years of mtDNA evolution before the sister species would be expected to exhibit reciprocal monophyly. Given a standard molecular clock calibration of 2% sequence divergence per million years, this timeframe is consistent with the formation of major clades in *Ulocentra*. Under the assumptions of the Avise *et al.* (1984) model, most snubnose species would thus be expected to exhibit polyphyletic relationships with their sister species today.

Despite the recent estimates of divergence times between nominal snubnose taxa, each species exhibits its own unique set of mtDNA haplotypes, providing no direct evidence for current genetic exchange between species examined at our sample sites. The nominal taxa of snubnose darters thus appear to be evolving independently from each other and appear to constitute valid species under the Phylogenetic Species Concept of Cracraft (1983) and Davis (1997).

ACKNOWLEDGMENTS

We thank C. A. Adams, M. I. Carter, R. D. Clipson, C. M. Demko, M. Kibbey, W. J. Poly, J. W. Provance, IV, C. Roberts, and J. Studebaker for help with stream collections. Additional specimens were kindly contributed by M. Binkley, N. M. Burkhead, N. H. Douglas, D. A. Etnier, W. J. Poly, and S. J. Walsh. Technical laboratory consultation was given by G. Booton, W. Duan, J. Kreiger, W. Mwanja, M. Schug, D. Stothard, and L. Wu. Assistance with parsimony analyses and statistics was provided by J. W. Wenzel. Help with data manipulation, figures, and tables was contributed by G. Booton, C. Caprette, H. Caprette, and B. Mark. This study benefited from discussions with J. C. Avise, R. M. Bailey, D. A. Etnier, L. M. Page, W. J. Poly, R. D. Suttkus, and J. W. Wenzel. We also appreciated comments from two anonymous reviewers. This study was supported by the Ohio State University, Departments of Molecular Genetics and Zoology, an Alumni Research Award of the Ohio State University Graduate School, and an Ohio State University Presidential Fellowship to B.A.P.

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